

The Risk Evaluation of Tungsten Oxide Nanoparticles in Cultured Rat Liver Cells for Its Safe Applications in Nanotechnology

Hasan Turkez^{1,2*}, Erdal Sonmez^{2,3}, Ozlem Turkez⁴, Yousef Ibrahim Mokhtar⁵, Antonio Di Stefano⁶ and Guven Turgut³

¹Department of Molecular Biology and Genetics; Erzurum Technical University; Erzurum - Turkey. ²Department of Nanoscience and Nanoengineering; Advanced Materials Research Laboratory; Graduate School of Natural and Applied Sciences; Atatürk University; Erzurum - Turkey. ³Department of Physics, Atatürk University; Erzurum - Turkey. ⁴Department of Biology; Atatürk University; Erzurum - Turkey. ⁵Department of Environmental Studies; Alexandria University; Alexandria - Egypt. ⁶Dipartimento di Farmacia; Università "G. D'Annunzio"; Chieti - Italy

ABSTRACT

Tungsten (VI) oxide (WO₃) nanoparticles (NPs) are used for many industrial purposes in everyday life. However, their effects on human health have not been sufficiently evaluated. Therefore, the present study was designed to investigate the toxicity potentials of various concentrations (0 to 1000 ppm) of WO₃ NPs (<100 nm particle size) in cultured primary rat hepatocytes. The results of cell viability assay showed that the higher concentrations of dispersed WO₃ NPs (300, 500 and 1000 ppm) caused significant ($p < 0.05$) decreases of cell viability. Also, dose dependent negative alterations were observed in oxidative status and antioxidant capacity levels after the application of WO₃ in cultured rat primary hepatocytes. The results of genotoxicity tests revealed that these NPs did not cause significant increases of micronucleated hepatocytes (MNHEPs) but increased 8-oxo-2-deoxyguanosine (8-OH-dG) levels as compared to the control culture.

Key words: tungsten oxide, nanotoxicity, genotoxicity, cytotoxicity, hepatocytes, oxidative stress

INTRODUCTION

Nanotechnology has emerged to be one of the most powerful technology creating approaches in the past half a century. Nowadays, nanotechnology has spawned the development of a veritable plethora of novel nanoparticles for diverse applications, ranging from solar energy capture to cosmetics and drug delivery (Riehemann et al. 2009; Patel and Patel 2013). With frequent exposure to dispersed nanoparticles from the composite products or workplaces, there is an increased chance for nanoparticles or nano

composites to enter human body and to relocate in metabolism-active organs (Song et al. 2009). Thus, studying the toxicity of nanomaterials is of importance to provide the guidance to occupational health and safety (Li et al. 2008; Lanone et al. 2009; Turkez et al. 2013a). The discussion about safety concerns associated with small particles is ongoing for many decades and, to a large extent, is related to the potential risks following inhalative, oral, parenteral or dermal exposure (Schmid et al. 2009). Especially, ultrafine or NPs are in the focus of the debate, e.g.

* Author for correspondence: hasanturkez@gmail.com

(ECETOC 2005), usually meant to have one or more size dimensions between 0.1 and 100 nm.

In last twenty years, many efforts were made to investigate the toxicity of micro sized natural and man-made mutagens human life and the ability of therapeutic substances on reducing the toxicity of these various toxicants (Turkez et al. 2005; Geyikoglu et al. 2005; Turkez and Geyikoglu 2005; Turkez and Sisman 2007; Turkez et al. 2007; Sisman and Turkez 2010; Rodhger et al. 2012; Turkez and Aydin 2013; Bupesh et al. 2013). But the toxic effects of nano sized particles have not fully studied, except for some inorganic and organic NPs. In fact, a recent report indicated that there was a lack of systematic assessment of the DNA damaging and carcinogenic potential of NPs in spite of their extensive use in nanotechnological applications (Turkez et al. 2013b).

Tungsten (VI) oxide (or tungsten trioxide: WO_3) is a chemical compound containing oxygen and the transition metal tungsten. It is obtained as an intermediate in the recovery of tungsten from its minerals. Tungsten ores are treated with alkalis for its production. WO_3 occurs naturally in the form of hydrates such as tungstite ($WO_3 \cdot H_2O$) and meymacite ($WO_3 \cdot 2H_2O$). WO_3 is used for many purposes in everyday life. It is frequently used in industry to manufacture tungstates for x-ray screen phosphors, for fireproofing fabrics and in the production of electro chromic windows and gas sensors. Due to its rich yellow color, it is also used as a pigment in ceramics and paints (Erik and Wolf-Dieter 1999; Lee 2000; Pradyot 2003; Merck Index 2006). Tungsten is also used in many military applications since it has the second highest melting temperature of any element. It is generally considered that elucidating the potential health effects of tungsten is important and necessary (Witten et al. 2012). Tungsten (as sodium tungstate) has been found to accumulate in several organs and/or tissues such as kidneys, liver, ovaries, uterus, prostate, pancreas, lung, heart, muscle, spleen and bone following a single oral dose (McInturf et al. 2011). Furthermore neurotoxic (Shan et al. 2012) and dermal toxic (Zhang et al. 2010) effects by tungsten contained NPs have also been reported.

In recent years, tungsten containing nanomaterials due to their big surfaces have attracted a dramatic and exponentially increasing interest in nanotechnological products (Zhou et al. 2012).

Thus, possible health impact of WO_3 NPs upon introduction into the body is of great interest. With the increased applications of WO_3 NPs, the concerns about their potential human toxicity effects and their environmental impact have also increased. Cytotoxicity, inflammation and increased oxidative stress through reactive oxygen species (ROS) formation are prominently discussed to be relevant factors regarding the safety of small particles down to the nano-range (Knaapen et al. 2004; Unfried et al. 2007; Lewinski et al. 2008; Turkez 2008 and 2011). It has been reported that different sizes and morphologies of NPs have the potential to influence the interaction with many kind of biomolecules, including proteins, enzymes and DNA (Grandjean-Laquerriere et al. 2005; Ramesh et al. 2007; Xu et al. 2012). The liver was considered as a target site for nanotoxicity due to its accumulative properties after ingestion, inhalation or absorption (Wang et al. 2011). However, recorded hepatotoxicity data for WO_3 NPs relating to human health are very scarce. Since this is considered to be of particular importance for the investigation of mechanisms of ROS formation and oxidative stress, in the present study, specific measurements were performed in the cultured rat primary hepatocytes as *in vitro* model system for assessing the impact of WO_3 NPs on human and environmental health.

MATERIALS AND METHODS

Synthesis of WO_3 NPs

Li_2TiO_3 powder was synthesized via sol-gel route (Poulter and Pryde 1968; Maruyama et al. 1994; Han et al. 1995; Sofian et al. 2007; Ilcan et al. 2008; Ghodsi and Absalan 2010) WO_3 nanopowders (<100 nm) were prepared by sol-gel process. The experimental details are shown in Figure 1. Firstly, nitric acid (HNO_3) solution was added drop-by-drop to sodium tungstate ($Na_2WO_4 \cdot 2H_2O$). Oxalic acid ($H_2C_2O_4$) and citric acid ($C_6H_8O_7$) were used as complex agents at sol solution. The precipitate obtained was washed several times with absolute ethyl alcohol and dried at 50 °C. The yellow precipitate (WO_3) powder was produced with calcination at 550 °C for 3h. All the chemicals were of analytical grade and supplied by Sigma/Merck.

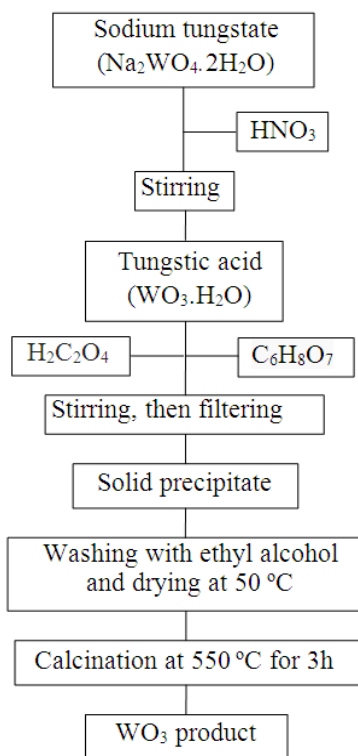


Figure 1 - Schematic diagrams of steps involved in obtaining WO_3 powders.

Animals

Male rats of Sprague-Dawley strain (from Medical Experimental Research Center, Atatürk University, Erzurum, Turkey) of 200-300 g body weight, were used throughout the present studies. They were allowed water and standard laboratory chow ad libitum and were maintained under the standard light, temperature, and relative humidity conditions. The study protocol was approved by the local ethical committee. All the experiments were performed in accordance with the Guide for the Care and Use of Laboratory Animals.

Hepatocyte isolation and cultivation

Rats were sacrificed by CO_2 overdose and the livers were removed immediately. Isolated hepatocytes from the rats were prepared by the collagenase perfusion technique. The liver was perfused through the hepatic portal vein with calcium-free Hanks balanced salt solution to remove the blood for about 10 min at a flow rate of 2.5 mL min^{-1} . As soon as the liver became grayish brown in color, a second buffer solution containing collagenase (Hank's balanced salt supplemented with 4 mM calcium chloride and $0.5 \text{ mg collagenase mL}^{-1}$) was perfused at the same

rate until the liver appeared to have broken up. Then the liver was into 3-4 mm pieces with a sterile scalpel. Following the mechanical dissociation, the cells were filtered through the gauze and centrifuged at 250 xg for 5 min. Then, the hepatocytes were collected in the medium containing bovine serum albumin and bovine insulin. The cell suspension was filtered through the gauze again and allowed to sediment for 20 min to eliminate cell debris, blood, and sinusoidal cells. The cells were then washed three times by centrifugation at 50 g and tested by Trypan blue dye exclusion for the viability (always in the range of 82-93%). The hepatocytes were then suspended in a mixture of 75% Eagle's minimum essential medium and 25% medium 199, supplemented with 10% fetal calf serum containing streptomycin, penicillin, bovine insulin, bovine serum albumin and NaHCO_3 (2.2 mg). The hepatocytes were plated in multi-well tissue culture plates (3×10^5 cells in a well area of 3.8 cm^2 ; 8×10^5 cells in a well area of 9.6 cm^2). The medium was changed 3-4 h later. The effect of WO_3 NPs was studied after 72 h of exposure in the cultures maintained with a medium deprived of fetal calf serum but supplemented with hydrocortisone hemisuccinate ($7 \times 10^{-7} \text{ M}$). Hepatocytes were cultured for an additional 8 h before the treatment.

Treatments

After 8 h of plating, when primary hepatocytes were adhered and attained their epithelial morphology, culture medium was aspirated and replaced with an equal volume of the medium supplemented with different concentrations of aqueous WO_3 NPs (5, 10, 20, 50, 75, 100, 150, 300, 500 and 1000 ppm) followed by incubation in CO_2 incubator for 72 h ($n=6$). Mitomycin C (MMC; $\text{C}_{15}\text{H}_{18}\text{N}_4\text{O}_5$; Sigma®, at 10^{-7} M) added group was considered as positive control (Control⁺).

MTT assay

The viability of the cells was assessed by measuring the formation of a formazan from 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) spectrophotometrically test, modified after Mosmann (1983). Hepatocytes were incubated with 0.7 mg mL^{-1} MTT for 30 min at 37°C at the end of the experiment. After washing with PBS, the blue formazan was extracted from the cells with isopropanol/formic acid (95:5) and was

photometrically determined at 560 nm (Lewerenz et al. 2003).

LDH assay

Lactate dehydrogenase (LDH) activity was measured in the culture medium as an index of cytotoxicity, employing an LDH kit (Bayer Diagnostics®, France) adapted to the auto analyzer (ADVIA 1650, USA). Enzyme activity was expressed as the extracellular LDH activity percentage of the total activity on the plates.

Total antioxidant capacity and total oxidant status assays

The automated Trolox equivalent total antioxidant capacity (TAC) and total oxidant status (TOS) assays were carried out in the culture medium by commercially available kits (Rel Assay Diagnostics®, Gaziantep, Turkey).

LMN assay

Liver micronucleus assay (LMN) assay was done by using the method of Suzuki et al. (2009). Immediately prior to the evaluation, 10-20 μL of hepatocyte suspension was mixed with an equal volume of acridine orange (AO)-DAPI (4',6-diamidino-2-phenylindole dihydrochloride) stain solution (AO, 0.5 mg mL^{-1} ; DAPI, 10 $\mu\text{g mL}^{-1}$) for fluorescent staining. Approximately 10-20 μL of the mixture was dropped onto a glass slide and covered with a cover glass. Samples of well-isolated hepatocytes were evaluated with the aid of a fluorescence microscope counting the number of MNHEPs in 2000 hepatocytes for each animal. MNHEPs were defined as hepatocytes with round or distinct MNs that stained like the nucleus, with a diameter 1/4 or less than that of the nucleus, and confirmed by focusing up and down, taking into account hepatocyte thickness by one observer.

Nucleic acid oxidation

DNA oxidation was determined by measuring the amount of 8-OH-dG adducts. DNA was digested by incubation with DNAase I, endonuclease, and alkaline phosphatase (Schneider et al. 1993). The amount of 8-OH-dG was measured by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Floyd et al. 1993).

Statistical analysis

The experimental data were analyzed using one-way analysis of variance (ANOVA) and Fischer's least significant difference (LSD) tests to

determine whether any treatment significantly differed from the controls or each other's. Results were presented as the mean \pm SD values and the level of 0.05 was regarded as statistically significant.

RESULTS

The results of cell viability measured by the MTT assay are shown in Figure 2. When assayed *in vitro* on the hepatocyte cells using the MTT assay, the value for the MMC-treated cells (as control⁺) was approximately 2.4-fold lower than that for the control. Similarly, the higher concentrations of WO_3 NPs (300, 500 and 1000 ppm) caused significant ($p < 0.05$) decreases of the cell viability. However, the hepatocyte cells exposed to lower doses than 300 ppm of WO_3 NPs did not show any significant change in cell viability during 72 h as determined by the MTT assay. No cytotoxicity was observed for the control⁻ cells. MMC-induced hepatic damage was clearly evidenced by 6-fold increases in the activity of LDH compared with the observations of the negative controls (Fig. 3). Although LDH was not affected by the low doses of WO_3 NPs alone, but the increases of the levels of enzyme reached statistical significances at 300, 500 and 1000 ppm.

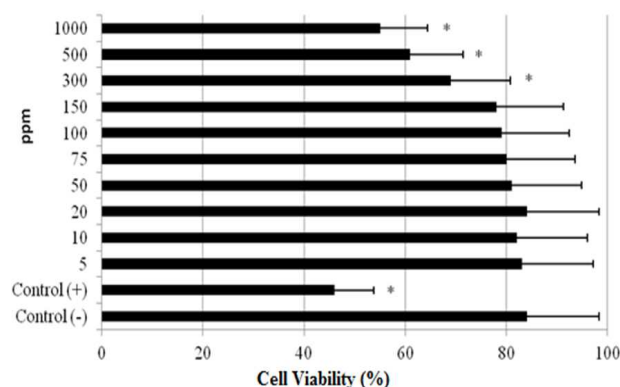


Figure 2 - MTT reduction [3-(4,5-dimethyl-thiazol-2-yl) 2,5-diphenyltetrazolium bromide] in cultured rat hepatocytes maintained in the presence of different WO_3 NPs concentrations for 72 h.

(Each individual hepatocyte culture without NPs was studied as a negative control group (Control⁻). MMC alone added group was considered as a positive control (Control⁺). Values are means \pm standard deviation ($n = 6$). * symbol presents significant differences at the $p < 0.05$ level from the control⁻ group).

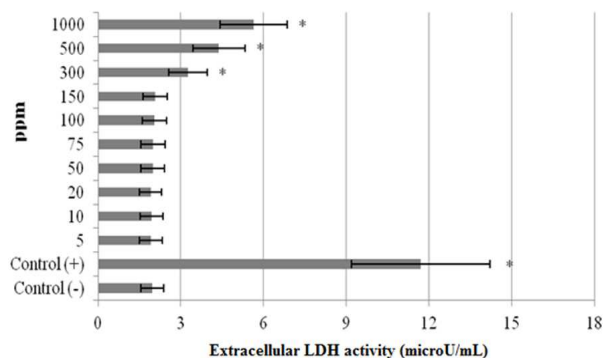


Figure 3 - Extracellular level of lactate dehydrogenase (LDH) in cultured rat hepatocytes maintained in the presence of different WO₃ NPs concentrations for 72 h. Each individual hepatocyte culture without NPs was studied as a negative control group (Control -). MMC alone added group was considered as a positive control (Control +). Values are means \pm standard deviation (n = 6). * symbol presents significant differences at the p<0.05 level from the control - group).

The levels of 8-oxo-2-deoxyguanosine (8-OH-dG) in the cultured rat hepatocytes of controls and experimental groups are shown in Figure 4. Firstly, the levels of 8-OH-dG, a sensitive marker of oxidative DNA damage, were quantified with regard to MMC treatment. It was observed that MMC significantly increased 8-OH-dG concentrations in the hepatocyte cultures after 72 h. Similarly, 8-OH-dG levels increased in the hepatocyte cells that were treated with 500 and 1000 ppm of WO₃ NPs.

The results of the observed LMN rates in the primary rat hepatocyte cells after 72 h WO₃ NPs treatment are presented in Figure 5. LMN analyses did not show statistically significant differences (p>0.05) between the control and tested NPs applied cultures.

Table 1 showed the effects of WO₃ NPs on oxidant status in the cultured rat hepatocytes, which were determined by the TAC and TOS analysis. The TAC value decreased with the addition of MMC while TOS value increased. In contrast to the treatments with 5, 10, 20, 50, 75 and 100 ppm of WO₃ NPs did not alter the TAC and TOS levels. However, nanomaterials applications at higher doses (150, 300, 500 and 1000 ppm) changed the TAC and TOS levels. Thus, WO₃ NPs had dose dependent effects on the oxidative damage in hepatocytes *in vitro*.

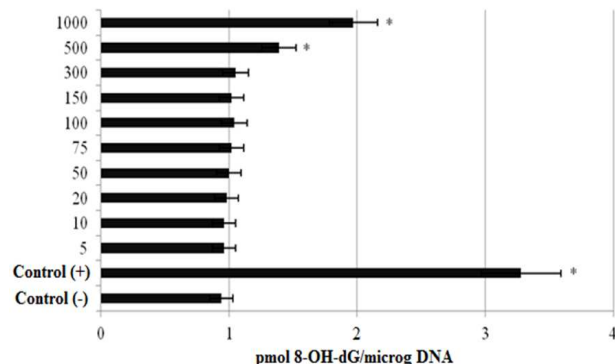


Figure 4 - 8-oxo-2-deoxyguanosine (8-OH-dG) adducts in cultured rat hepatocytes maintained in the presence of different WO₃ NPs concentrations for 72 h. Each individual hepatocyte culture without NPs was studied as a negative control group (Control -). MMC alone added group was considered as a positive control (Control +). Values are means \pm standard deviation (n = 6). * symbol presents significant differences at the p<0.05 level from the control - group).

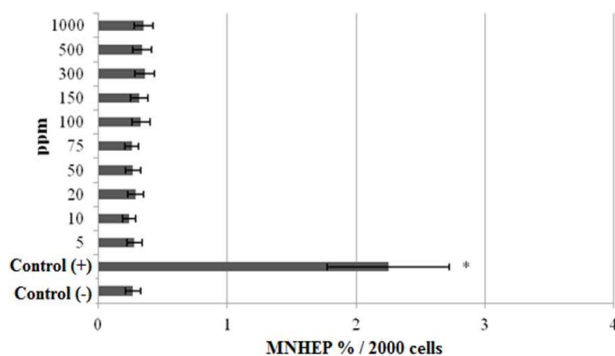


Figure 5 - Results of liver MN assay in cultured rat hepatocytes maintained in the presence of WO₃ NPs for 72 h. HEP = hepatocyte; MNHEP = Number of micronucleated hepatocytes. Each individual hepatocyte culture without NPs was studied as a negative control group (Control -). MMC alone added group was considered as a positive control (Control +). Values are means \pm standard deviation (n = 6). * symbol presents significant differences at the p<0.05 level from the control - group).

Table 1 - Extracellular TAC and TOS levels in cultured rat hepatocytes maintained with WO₃ NPs for 72 h. Each individual hepatocyte culture without NPs was studied as a negative control group (Control -). Ascorbic acid (10 μM) and hydrogen peroxide (25 μM) added groups were also used as the control+ in TAC and TOS analysis, respectively. Values are means ± standard deviation (n = 6). * symbol presents significant differences at the p<0.05 level from the control - group).

Treatment	TAC (mmol Trolox Equiv./l)	TOS (μmol H ₂ O ₂ Equiv./l)
Control ⁻	3.9 ± 0.9	9.1 ± 2.9
Control ⁺	2.1 ± 0.7*	16.5 ± 3.4*
5 ppm	3.9 ± 0.8	9.3 ± 3.3
10 ppm	3.8 ± 0.9	9.2 ± 3.4
20 ppm	3.8 ± 1.1	9.4 ± 3.7
50 ppm	3.6 ± 1.1	9.6 ± 4.0
100 ppm	3.4 ± 1.3	9.6 ± 3.6
150 ppm	3.1 ± 0.9*	10.8 ± 4.1*
300 ppm	2.7 ± 1.3*	12.9 ± 4.2*
500 ppm	2.6 ± 0.7*	13.4 ± 4.5*
1000 ppm	2.6 ± 1.1*	14.2 ± 4.4*

DISCUSSION

In the present investigation, it was aimed to evaluate the cytotoxicity, genotoxicity and oxidative damage in the cultured rat healthy hepatocyte cells in response to different concentrations of WO₃ NPs. Trends in the cytotoxicity as observed for the different concentrations of these NPs were overall highly similar using the two independent assays (MTT and LDH) in the cultured rat hepatocytes. A particular contrast was observed for the highest concentrations of WO₃ NPs, which induced a pronounced LDH release and decrease of MTT. As a matter of fact, tungsten carbide nanoparticles (WC NPs) caused reduction in cell viability between 10 and 50% compared to controls upon particle exposure in the rainbow trout (*Oncorhynchus mykiss*) gill cell line, RTgill-W1 (Kühnel et al. 2009). Rothen-Rutishauser et al. (2007) reported that the size of nano-particles or their aggregates could be considered a potential determinant for the uptake and subsequent macrophage responses, which could explain the observed differences on LDH release at the highest concentration of NPs (Albrecht et al. 2009). Particles in the fine size range may induce more pronounced responses than in the nano-range (Rothen-Rutishauser et al. 2007), and the “ultrafine hypothesis” has been challenged

previously by the experimental studies (Schins et al. 2004). Albrecht et al. (2009) found that cytotoxicity as observed at high concentrations did not necessarily represent a compound-related effect, and could at least partly be due to physical coverage of the cells by the test NPs.

The fully understanding of how WO₃ NPs interact with hepatocytes, especially at the molecular level, is still unclear. Present findings demonstrated that direct exposure of the hepatocytes to WO₃ NPs induced the intracellular ROS generation, reduced the total antioxidant capacity (TAC), increased 8-OH-dG levels, and subsequently caused dysfunction of these cells. In accordance with the present results, it has been reported that engineered nanomaterials, either metals (like carbon and silver) or metal oxides (like zinc oxide, magnesium oxide, and titanium oxide), induce toxicity and oxidative stress by generating free radicals (Turkez and Geyikoglu 2007; Anreddy et al. 2013). Mechanisms involved in the reduction of cell viability through oxidative stress by WO₃ NPs are not yet known. Oxidative stress is associated with protein and lipid oxidation, ultimately leading to a profound alteration in mitochondrial function (Pan et al. 2009). Any change in the mitochondrial membrane permeability is known to be an early event in apoptosis (Liu and Sun 2010). It is known that ROS activates multiple signaling pathways, including mitogen activated protein kinase (MAPK) family and p53 expression signal transduction cascades (Simbula et al. 2007). P53, Bax and Bcl-2 are also generally thought to be involved in mitochondria-dependent apoptosis (Liu and Sun 2010). p-JNK, p-c-Jun, p-p53, Bax, cleaved caspase-3 were significantly increased in the human umbilical vein endothelial cells (HUVECs) after exposure to silica nanoparticles, while Bcl-2 was dramatically suppressed. ROS scavenger could markedly inhibit the JNK, c-Jun and p53 activity, indicating that ROS could be upstream effectors of JNK and p53 (Liu and Sun 2010). Hsin et al. (2008) found that nano silver-induced apoptosis was mediated by the ROS via JNK and p53 activation. ROS could also directly activate p53, most probably by the induction of DNA damage (Simbula et al. 2007; Turkez and Togar 2010; Turkez and Geyikoglu 2010; Turkez et al. 2012). Based on the results of Liu and Sun (2010), the following silica nanoparticles-mediated signaling pathways for apoptosis related events were proposed as ROS production (JNK/c-Jun

phosphorylation), P53 activation, Bax up regulation and Bcl-2 down regulation.

Previous studies showed that higher dose exposure to nanomaterials, including silica nanoparticles, titanium dioxide, zinc oxide, alumina nanoparticles led to proinflammatory and procoagulant responses in endothelial cells. It is an accepted view point that ROS are involved in many of the processes underlying endothelial activation (e.g., the up regulation of adhesion molecules and chemokines, increased expression of tissue factor). Many of the key signal transduction molecules involved in the endothelial activation, such as various MAPKs and the transcription factors NF- κ B, are known to be redox sensitive (Alom-Ruiz et al. 2008). To assess the biological effect of different concentrations of WO₃ NPs on rat hepatocyte cells, cell viability was determined. In the present study, MTT and LDH assays showed that the higher concentrations of WO₃ NPs decreased cell viability. Also, Liu and Sun (2010) found that exposure to high concentrations (100-200 ppm) of silica nanoparticles caused an increase of cytotoxicity in HUVECs. The LDH release was also increased by silica nanoparticles only at the highest concentration, indicating that exposure to high concentrations of silica nanoparticles could affect cell-membrane integrity and lead to cell death. They also reported the induction of apoptosis or necrosis by silica nanoparticles and oxidative stress as silica nanoparticles induced toxicity mechanism. Liu and Sun (2010) showed that intracellular ROS generation of HUVECs by silica nanoparticles was gradually increased in a time- and dose-dependent manner, suggesting that oxidative stress occurred not at once, but continuously during the cell culture. ROS played a central role in silica nanoparticles-mediated apoptosis. Disturbance of membrane integrity has been recently suggested as possibly one of the mechanisms for cytotoxic action in the nanoparticles (Kim et al. 2009). In accordance to the present findings, the results by Ding et al. (2009) had demonstrated that nano-WC-Co generated a higher level of hydroxyl radicals, induced greater oxidative stress, as evidenced by a decrease of glutathione (GSH) levels.

Present results indicated that WO₃ NPs did not induce the LMN formations in *in vitro* conditions. The study of DNA damage at the chromosome level is an essential part of genotoxicity testing because chromosomal mutation is an important

event in carcinogenesis. LMN assays have emerged as one of the preferred methods for assessing the chromosome damage because they enable both chromosome loss and chromosome breakage to be measured reliably. The NPs, which were located in the cytosol near the nucleus but not were found inside the nucleus, in mitochondria or ribosomes did not induce DNA-breakage (Bhattacharya et al. 2009). But WO₃ NPs led to oxidative DNA damages as determined by the increases of 8-OH-dG levels, which were the major products of DNA oxidation in present study. Thus, apparently WO₃ NPs were not clastogenic but were able to generate elevated amounts of free radicals, which induced indirect genotoxicity, mainly by DNA-adduct formation. In contrast to our *in vitro* findings, WO₃ NPs showed positive mutagenic response in TA1537 and TA98 bacterial strains of *Salmonella typhimurium* by using Ames test (Hasegawa et al. 2012). WO₃ NPs did not cause increase of the incidence of chromosome aberrations in rat bone marrow cells but led to increases of MN formation after chronic exposure for 30 days (Turkez et al. 2013a). These conflicting evidences on genotoxicity by WO₃ NPs could be explained by the differences in conditions (*in vivo* or *in vitro*), exposing time (acute, chronic or sub chronic) and chemical features of tested NPs (exact size, shape, composition, and aggregation). It is known that studies dealing with nanotoxicity have focused on *in vitro* cell culture studies. However, data obtained from such studies might not correspond to *in vivo* results. Hence, a full *in vivo* life cycle characterization framework would be necessary for systematic evaluation of the size, shape, and surface chemistry of NPs, and their correlation to *in vivo* behavior. *In vivo* systems were extremely complicated and the interactions of NPs with biological components, such as proteins and cells could lead to unique bio-distribution, clearance, immune response, and metabolism (Fischer and Chan 2007; Berger 2008).

In summary, data from the current study showed that exposure to WO₃ NPs at high concentrations caused ROS generation and decreased the total antioxidant capacity (TAC) in cultured primary rat hepatocytes. NPs caused decreased cell viability, which was detected by the increased MTT and LDH release. The present findings also showed that WO₃ NPs had weak genotoxic potential *in vitro*. Overall, these findings suggested that

exposure to WO₃ NPs could pose environmental and human health risk.

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